

## **Isosilibinin Inhibits Advanced Human Prostate Cancer Growth in Athymic Nude Mice: Comparison with Silymarin and Silibinin**

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### **Abstract:**

Earlier studies have shown the cancer chemopreventive efficacy of silymarin and its semi-purified constituent silibinin against prostate cancer (PCa), but the efficacy of other constituents of silymarin is largely unknown. In the present study, we assessed the *in vivo* growth inhibitory efficacy of one such constituent isosilibinin (a 50:50 mixture of isosilybin A and isosilybin B) in comparison with silymarin and silibinin in human PCa DU145 xenograft in athymic nude mice. Isosilibinin feeding (200 mg/kg body weight per day) significantly inhibited the growth of xenograft after 53 days of treatment ( $p \leq 0.005$ ), which was equally or slightly better effective than silymarin and silibinin, respectively. Treatment with isosilibinin, silymarin and silibinin was stopped after 53 days and tumor volume was measured till 77 days. After 24 days of treatments withdrawal, tumor volume remain decreased, however, it was statistically significant only with isosilibinin ( $p \leq 0.05$ ), suggesting its prolonged effect. Biomarker analysis showed that isosilibinin, silymarin and silibinin treatment for 53 days significantly inhibited the immunoreactivity for proliferating cell nuclear antigen (PCNA), microvessel density (CD31) and vascular endothelial growth factor along with significant increase in apoptotic cell population. The PCNA levels in tumors remained significantly low even after 24 days of treatments withdrawal. Western blot analysis of tumor tissue suggested that these flavonolignan formulations differentially alter the expression of cell cycle regulatory molecules, cyclins and Cdks. Overall, the results of present study suggest that isosilibinin has comparatively better efficacy against PCa and should be further analyzed for its clinical utility.

**Keywords:** prostate cancer | isosilibinin | angiogenesis | cell cycle | apoptosis

## Article:

Prostate cancer (PCa) is the most commonly diagnosed malignancy among men in the western world including United States.<sup>1</sup> According to American Cancer Society report, about 218,890 incidences (29% of total estimated new cases) and 27,050 deaths (9% of total estimated deaths) due to PCa were estimated in American men in the year 2007.<sup>1</sup> In initial stages, PCa is androgen-dependent and treatment options include hormone ablation therapy, surgery, radio- and chemotherapy.<sup>2-4</sup> These treatments only delay the recurrence of the lethal hormone-refractory disease and patients have poor survival afterwards.<sup>3, 4</sup> For the late stage hormone-refractory PCa, chemotherapeutic drugs offer little survival benefit.<sup>3, 4</sup> Further, all these treatments have significant side effects including impotence and incontinence that adversely affect the quality of life for patients. These limitations of the current therapeutic regimens suggest the need for alternative strategies to decrease the morbidity and mortality due to this malignancy. In this regard, prevention and therapeutic intervention by phytochemicals has been suggested as a newer dimension in the arena of cancer management. Epidemiological and preclinical studies have also suggested the anticancer efficacy of phytochemicals, which are rich source of polyphenolic compounds.<sup>5-7</sup>

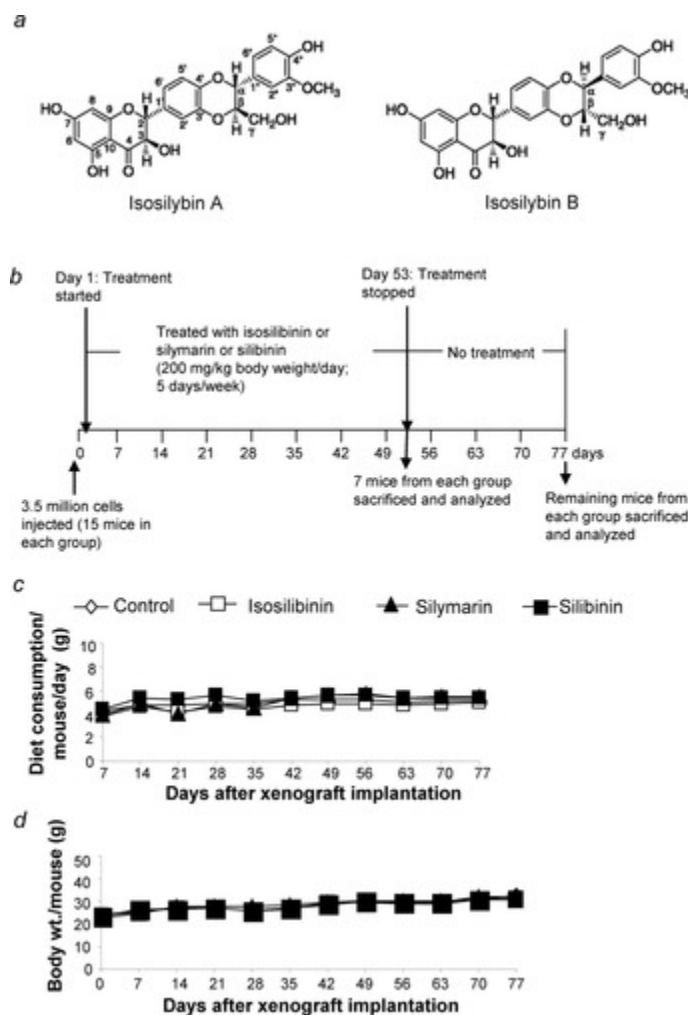
Silymarin is a crude mixture of polyphenolic compounds and is obtained mainly from milk thistle (*Silybum marianum*) and artichoke (*Cynara scolymus*), members of the Asteraceae family. Silymarin is widely known for its hepatoprotective property both in animal models of hepatic injuries and in humans.<sup>8-10</sup> In the last decade, anticancer efficacy of silymarin and its most active known constituent, silibinin, have been reported against various epithelial cancers namely skin, colon, liver, lung, bladder, prostate, breast, among others.<sup>11-15</sup> Despite these advances not much effort has been made to analyze the anticancer efficacy of other constituents of silymarin. Largely, these studies have examined milk thistle as either a complex mixture of at least 8 compounds, termed “silymarin,” which is a series of 7 structurally-related flavonolignans and the flavonoid, taxifolin<sup>16</sup> or as an approximately equal mixture of the 2 most prominent flavonolignans, termed silibinin, which consists of the diastereoisomers, silybin A and silybin B.<sup>16-18</sup> The focus of these studies was the *in vivo* effect of isosilibinin, which consists of the diastereoisomers, isosilybin A and isosilybin B<sup>16</sup> relative to silymarin and silibinin (Fig. 1a). The nomenclature of silymarin and related constituents is quite complex and has been reviewed recently.<sup>16</sup>

We have reported that these pure compounds from silymarin have differential anticancer effects on various biological and molecular endpoints.<sup>18, 19</sup> In general, isosilybin B and isosilybin A were found to be most effective in inhibiting the cell survival, prostate specific antigen (PSA) secretion, androgen receptor level and topoisomerase II $\alpha$  promoter activity in human PCa cells.<sup>18, 20</sup> Further studies have suggested that these 2 compounds significantly inhibit the cell growth *via* modulating cell cycle regulatory molecules in human PCa LNCaP and 22Rv1 cells.<sup>21</sup> These *in vitro* studies in androgen-dependent and androgen-independent PCa cells clearly suggest the superior efficacy of the isosilibinin fraction relative to other constituents of

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Abbreviations: CD31, cluster of differentiation 31; CDK, cyclin-dependent kinase; Isosil, Isosilibinin; PCa, prostate cancer; PCNA, proliferating cell nuclear antigen; RPMI, Roswell Park Memorial Institute; SB, silibinin; SEM, standard error of mean; SM, silymarin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; VEGF, vascular endothelial growth factor.

silymarin. Therefore, the present study was designed, for the first time, to examine the *in vivo* efficacy of isosilibinin against DU145 hormone-independent human prostate xenograft growth in nude mice, and its comparison with silymarin and silibinin. Additionally, we examined the effect of these 3 formulations on *in vivo* biomarkers for proliferation, apoptosis and angiogenesis along with associated alterations in the expression of cell cycle regulatory molecules in tumor tissue. We also monitored the effect of treatments withdrawal on tumor growth kinetics. The findings of the present study identified *in vivo* anti-PCa efficacy of isosilibinin which was relatively better than silibinin and silymarin.



**Figure 1.** Effect of isosilibinin, silymarin and silibinin treatments on the body weight and diet consumption by athymic nude mice. (a) Chemical structure of isosilybin A and isosilybin B. (b) Experimental design for the xenograft experiment. (c) Food intake was recorded throughout the experiment and the diet consumption/mouse/day (g) is plotted as a function of time (days) for each group. Each point is a mean value of 14–15 mice till 53 days and 6–8 mice during 53–77 days. (d) Body weight of each mouse in different groups was recorded twice a week throughout the experiment and is plotted as a function of time (days) for each group. Each point is a mean value of 14–15 mice till 53 days and 6–8 mice during 53–77 days.

## Material and methods

### Cell line and reagents

DU145 human prostate carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 95% air and 5% CO<sub>2</sub> atmosphere. DU145 cells grown as monolayer were harvested by brief incubation with 0.25% trypsin-EDTA solution and used for xenograft implantation in nude mice. RPMI 1640 media and other cell culture materials were from Invitrogen Corporation (Gaithersburg, MD). Matrigel was from BD Biosciences (New Bedford, MA). TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay kit was from Promega Corporation (Madison, WI). Harris hematoxylin, carboxymethylcellulose (CMC), 3,3'-diaminobenzidine (DAB) and antibody for β-actin were from Sigma (St. Louis, MO). Streptavidin, primary antibody for proliferating cell nuclear antigen (PCNA) and biotinylated anti-mouse secondary antibody were from Dako (Carpinteria, CA). Primary antibody for CD31 was from Abcam (Cambridge, MA). Antibodies for Cdk2, Cdk4, Cdk6, cyclin D1, cyclin D3, cyclin E, cyclin A and vascular endothelial growth factor (VEGF) were from Santa Cruz Biotechnology (Santa Cruz, CA). Normal goat serum and biotinylated anti-rabbit secondary antibody were also from Santa Cruz Biotechnology (Santa Cruz, CA). Silymarin was obtained as a powdered extract (1 kg; Product No. 345066, Lot No. 37501) of the seeds (achenes) of *Silybum marianum* (L.) Gaertn. from Euromed, S.A. (Barcelona, Spain), which is a part of the Madaus Group (Cologne, Germany). The purification of silibinin (a 49:51 mixture of silybin A and silybin B, respectively) and isosilibinin (a 49:51 mixture of isosilybin A and isosilybin B, respectively) from silymarin has been described in detail.<sup>22</sup> Briefly, chromatographic fractions enriched in the compounds of interest were pooled until the ratio of the 2 desired diastereoisomers was as noted. Final purity was determined by HPLC as described,<sup>22</sup> and each sample was >99.5% pure.

#### Animals and diet

Athymic (*nu/nu*) male nude mice were obtained from the National Cancer Institute (Bethesda, MD) and housed in our animal care facility at standard laboratory conditions (in laminar airflow cabinets under pathogen-free conditions with a 12 hr light/12 hr dark schedule) and fed autoclaved Harlan Teklad Sterilizable rodent diet and water *ad libitum*. All the protocols used were approved by the Institutional Animal care and Use Committee of the University of Colorado Denver.

#### Experimental design for tumor xenograft study

Athymic nude mice were kept in the animal house facility for 1 week for acclimatization. To determine the effect of isosilibinin, silymarin and silibinin on prostate tumor growth, DU145 tumors were grown subcutaneously (s.c.) in nude mice. About 3.5 million DU145 cells were suspended in 0.05 ml of serum-free medium (RPMI), mixed with 0.05 ml of matrigel and were s.c. injected in the right flank of each mouse to initiate tumor growth (Fig. 1b). The following day after xenograft implantation, nude mice were randomly divided in to 4 groups: Group I was treated with 200 µl of 0.5% CMC (vehicle control); Group II was treated with 200 mg/kg body weight of isosilibinin (200 µl in 0.5% CMC); Group III was treated with 200 mg/kg body weight of silymarin (200 µl in 0.5% CMC); Group IV was treated with 200 mg/kg body weight of silibinin (200 µl in 0.5% CMC). These treatments were through oral gavage route (5 days/week)

and initiated the day after xenograft implantation (day 0) (Fig. 1b). At the end of 53 days after xenograft implantation, 7 animals from each group were sacrificed with lethal ketamine injection and tumors were harvested and used for immunohistochemical analysis (Fig. 1b). The remainder of the animals in each group were left without any further treatment and monitored until 77 days and then sacrificed and tumor tissues analyzed (Fig. 1b). Throughout the experiment, food consumption and animal body weight were monitored twice weekly. Once the tumor xenograft started growing, their sizes were measured twice weekly in 2 dimensions using a digital caliper. The tumor volume was calculated by the formula:  $0.5236 L_1(L_2)^2$ , where  $L_1$  is long diameter, and  $L_2$  is short diameter. At the termination of these studies, each tumor was carefully dissected and weighed. All small tumors and a piece of large tumors were fixed and processed for immunohistochemical analysis. The rest of the tumor tissue was snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for immunoblot analyses.

#### Immunohistochemical detection of PCNA in tumors

Tumor samples were fixed in 10% buffered-formalin for 12 hr and processed conventionally. The paraffin-embedded tumor sections (5  $\mu\text{m}$ -thick) were heat immobilized, deparaffinized using xylene, and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval was performed using 10mM citrate buffer (pH 6.0) in a microwave for 15 min at full and 20% power levels, respectively. Endogenous peroxidase activity was blocked by immersing the sections in 3.0%  $\text{H}_2\text{O}_2$  in methanol (v/v), followed by 3 changes in 10 mM PBS (pH 7.4). The sections were then incubated with mouse monoclonal anti-PCNA antibody (1:400) for 2 hr at room temperature in a humidity chamber and overnight at  $4^{\circ}\text{C}$ . Negative controls were treated only with PBS under identical conditions. The sections were then incubated with biotinylated rabbit anti-mouse antibody (1:200) for 1 hr at room temperature. Thereafter, following wash with PBS, sections were incubated with conjugated horseradish peroxidase-streptavidin for 45 min at room temperature in a humidity chamber. The sections were then incubated with DAB working solution for 5 min at room temperature, counterstained with diluted Harris hematoxylin for 2 min, and rinsed in Scott's water. Finally, proliferating cells were quantified by counting the PCNA-positive cells and the total number of cells at 5 arbitrarily selected fields at  $400\times$  magnification. The proliferation index (per  $400\times$  microscopic field) was determined as number of PCNA-positive cells  $\times$  100/total number of cells.

#### *In situ* apoptosis detection by TUNEL staining

Paraffin-embedded, 5  $\mu\text{m}$ -thick sections were also used to identify apoptotic cells by staining using TUNEL assay kit as per vendor's protocol. The extent of apoptosis was evaluated by counting the TUNEL-positive cells (brown-stained) as well as the total number of cells in 5 randomly selected fields at  $400\times$  magnification. The apoptotic index was calculated as (number of apoptotic cells  $\times$  100)/total number of cells.

#### Immunohistochemical analysis of tumors for CD31 expression

The staining procedure for CD31 (an endothelial cell-specific antigen also known as platelet endothelial cell adhesion molecule-1) was similar to that of PCNA staining with some modifications. Before incubation with primary antibody, tumor section were incubated with

normal goat serum for 30 min. Tumor sections were incubated for 2 hr at room temperature and overnight at 4°C with goat anti-rabbit CD31 polyclonal antibody (1:50). Then sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:200) followed by streptavidin-conjugated horseradish peroxidase (1:300). Antigen-antibody complexes were visualized by incubation with DAB substrate and counterstained with diluted Harris hematoxylin. Microvessels stained with CD31 (brown) were quantified in 5 random microscopic (400× magnification) fields per tumor.

#### Immunohistochemical analysis of tumors for VEGF expression

The staining procedure for VEGF was similar to that of PCNA staining using specific primary antibodies. Briefly, tumor sections were incubated with rabbit anti-VEGF (1:100) followed by incubation with appropriate biotinylated secondary antibody (1:200) and streptavidin (1:200). Finally, antigen-antibody complexes were visualized by peroxidase reaction with DAB substrate and counterstained with diluted Harris hematoxylin. VEGF immunoreactivity (represented by brown staining) was analyzed in 5 random areas for each tumor tissue and was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), 4+ (very strong staining).

#### Immunoblot analysis of tissue lysates

Total tissue lysates were prepared as described earlier.<sup>11</sup> Protein concentration in tissue lysates was determined with Bio-Rad detergent-compatible protein assay kit (Bio-Rad Laboratories) by the Lowry method, and 50–70 µg of protein/sample was then subjected to immunoblot analysis as described previously.<sup>11</sup> Membranes were probed for the protein abundance of desired molecules using specific primary antibodies followed by the appropriate peroxidase-conjugated secondary antibody and visualized by ECL detection. To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody, which was also used to normalize for differences in protein loading in densitometric analyses.

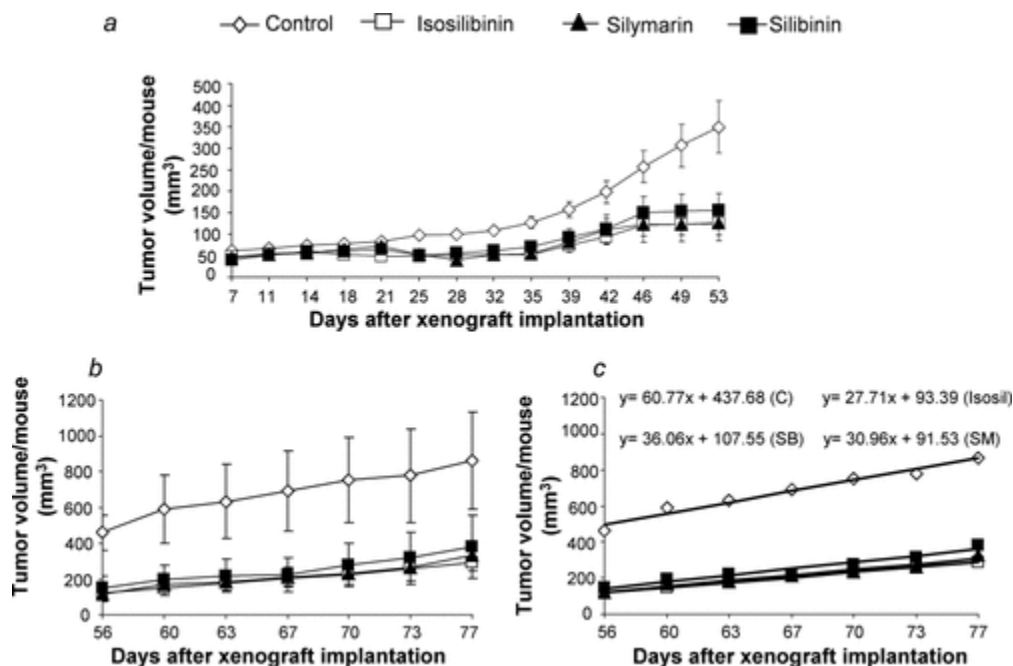
#### Immunohistochemical and statistical analyses

All the microscopic immunohistochemical analyses were performed using a Zeiss Axioscope 2 microscope (Carl Zeiss) and photographs were captured with a Carl Zeiss AxioCam MrC5 camera with Axiovision Rel 4.5 software. Western blots were scanned with Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA), and the mean density of each band was analyzed by the Scion Image program (National Institutes of Health, Bethesda, MD). In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific). Mean and SEM were used to describe the quantitative data. The statistical significance of difference between control and treated-groups was determined by Student's *t*-test or 1 way ANOVA followed by Bonferroni *t* test for pair-wise multiple comparisons and *p* < 0.05 was considered significant.

## Results

## General observations

At the time of necropsy, all animals were examined for gross pathology, and we did not observe any signs of abnormality in all the vital organs examined such as liver, lung, heart and kidney. The administration of isosilibinin, silymarin or silibinin (200 mg/kg body weight in CMC, 5 d/wk) through oral gavage did not cause any change in the diet consumption pattern of mice during the treatments (53 days) or after the withdrawal of treatments for 24 days (Fig. 1c). There were no significant differences between the mean body weights of mice in all the groups during the experimental period or during the withdrawal of the treatments (Fig. 1d). We also did not observe any significant difference among various groups in the ratio of prostate weight/body weight (data not shown).



**Figure 2.** Effect of isosilibinin, silymarin and silibinin treatments on DU145 tumor xenograft growth in athymic nude mice. Approximately 3.5 million DU145 cells were s.c. injected in the right flank of each mouse to initiate ectopic prostate tumor growth as described in “Material and methods”. The dose of isosilibinin or silymarin or silibinin was 200 mg/kg body weight. (a) Tumor volume in isosilibinin, silymarin and silibinin treated groups was measured twice weekly throughout the experiment using the formula:  $0.5236 L_1(L_2)^2$ . Each value represents mean and  $\pm$  SEM (error bars) of 14–15 mice for each group. (b) Treatment of isosilibinin, silymarin and silibinin was stopped after 53 days and tumor volume was measured twice weekly till 77 days. Each value represents mean and  $\pm$  SEM (error bars) of 6–8 mice for each group. The tumor volume data was also used for regression analysis after plotting a scatter diagram and the rate of tumor growth was measured from the equation shown in panel c. Abbreviations: C, control; Isosil, isosilibinin; SM, silymarin; SB, silibinin.

## Isosilibinin, silymarin and silibinin inhibit prostate cancer xenograft growth

All 3 formulations significantly inhibited the tumor volume during 53 days of treatment. Isosilibinin treatment resulted in significant decreases in mean tumor volumes 18 days onwards ( $p = 0.01$ ;  $n = 15$ ), whereas silymarin ( $n = 14$ ) and silibinin ( $n = 15$ ) treatment resulted in significant decreases in mean tumor volume only after 25 days of treatment ( $p \leq 0.001$ ;  $n = 15$ ) (Fig. 2a). After 53 days of treatment, isosilibinin treatment resulted in 64.10% decrease in the

tumor volume ( $p = 0.005$ ;  $n = 15$ ); silymarin treatment resulted in 63.64% decrease in the tumor volume ( $p = 0.01$ ;  $n = 14$ ); whereas silibinin treatment resulted in 55.47% decrease in the tumor volume ( $p = 0.05$ ;  $n = 15$ ) (Fig. 2a). These results showed the efficacy of these compounds in inhibiting the growth of PCa DU145 cells xenograft in nude mice.

Inhibitory effects of isosilibinin on tumor growth is more lasting compared to silymarin and silibinin

The treatments with isosilibinin, silymarin or silibinin were stopped after 53 days, and then 7 mice from each group was sacrificed, whereas the rest of the animals were monitored for tumor growth for 24 days without treatment. Even after 24 days of treatment withdrawal, there was 66.4% decrease in tumor volume with isosilibinin ( $p = 0.05$ ;  $n = 8$ ), and 61.8% with silymarin ( $n = 7$ ) and 55.6% with silibinin ( $n = 8$ ), but the data for silymarin and silibinin did not achieve statistical significance due to large variation in tumor size (Fig. 2b). The regression analysis showed that the rate of tumor growth was slowest in isosilibinin group followed by silymarin and silibinin (equations shown in Fig. 2c), but these differences in the rate of tumor growth did not achieve statistical significance.

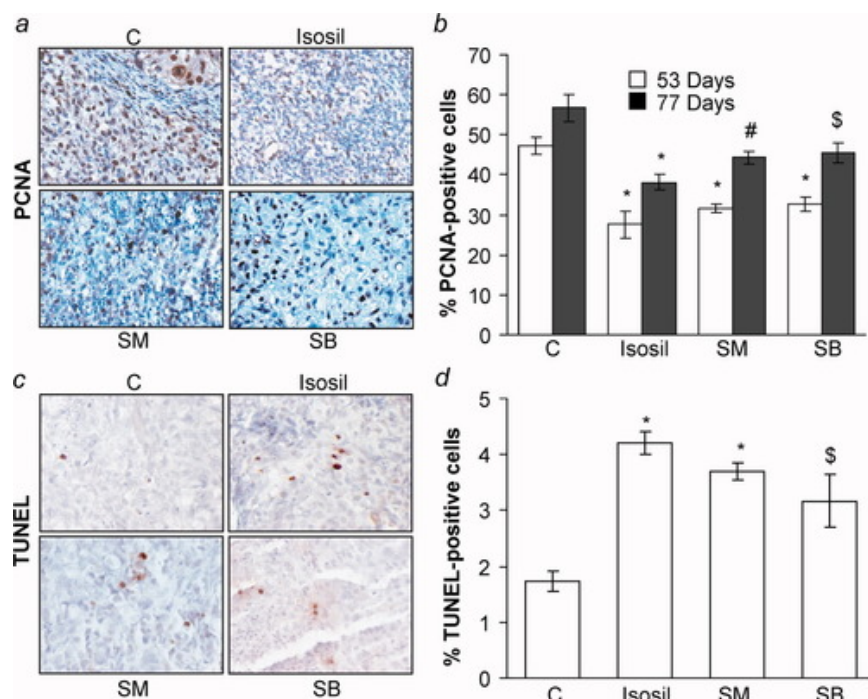
Isosilibinin, silymarin and silibinin inhibit proliferation of DU145 xenograft cells

Next, we analyzed the effect of these compounds on biochemical marker of proliferation in PCa xenograft tissue. The immunohistochemical analysis of tumor samples showed that the treatment of these compounds significantly inhibited the immunostaining for PCNA (Figs. 3a and 3b). Quantification of PCNA staining showed 41.8% ( $p \leq 0.001$ ) decrease in proliferation index with isosilibinin, 33.3% ( $p \leq 0.001$ ) decrease with silymarin treatment and 31.2% ( $p \leq 0.001$ ) decrease with silibinin treatment when compared with the control group after 53 days of treatment (Fig. 3b). However, the differences in the inhibitory effect of isosilibinin, silymarin and silibinin on PCNA staining were not statistically significant. Immunohistochemical analysis of the tumor tissues showed that even after 24 days of treatments withdrawal reduction in PCNA-positive cells in the isosilibinin group was 32.7% ( $p \leq 0.001$ ), the silymarin group 21.7% ( $p = 0.001$ ) and silibinin group 19.9% ( $p \leq 0.05$ ) relative to that of the control group (Figs. 3a and 3b). These results correlate with the decrease seen in the tumor volume even after withdrawal of these compounds.

Isosilibinin, silymarin and silibinin treatment induce apoptosis in DU145 xenografts

Next, we examined the effect of these compounds on apoptosis induction in xenograft tissue (after 53 days of treatment) by TUNEL staining. The results showed that isosilibinin treatment increased the apoptotic cell population by 2.4-fold ( $p \leq 0.001$ ), whereas silymarin and silibinin increased the apoptotic cell population by 2.1-fold ( $p \leq 0.001$ ) and 1.8-fold ( $p = 0.05$ ), respectively, when compared with the control group (Figs. 3c and 3d). There was however no significant difference in the apoptotic cell population between control and different treatment groups after 24 days of treatments withdrawal (data not shown).





**Figure 3.** *In vivo* anti-proliferative and pro-apoptotic effects of isosilibinin, silymarin and silibinin in DU145 tumor xenografts in nude mice. (a, b) At the end of 53 days and 77 days mice were sacrificed and tumor tissues were analyzed for immunohistochemical staining of proliferating cell nuclear antigen (PCNA) and photomicrographs were taken as detailed in the “Material and methods”. The representative images shown are from 77 days tumor tissue. Proliferation index was calculated as number of PCNA positive cells  $\times$  100/total number of cells counted under  $\times 400$  magnification in 5 randomly selected areas in each tumor sample. Statistical significance shown for isosilibinin, silymarin and silibinin is with respect to corresponding controls (53 or 77 days). (c, d) Apoptotic cell population in various groups after 53 days of treatment was measured by TUNEL assay as detailed in the “Material and methods”. Apoptotic index was calculated as number of positive cells  $\times$  100/total number of cells counted under  $\times 400$  magnification in 5 randomly selected areas in each tumor sample. The data shown in the bar diagram represents mean and  $\pm$  SEM (error bars) of 4–5 samples for each group. Abbreviations: C, control; Isosil, isosilibinin; SM, silymarin; SB, silibinin. \*,  $p \leq 0.001$ ; #,  $p \leq 0.01$ ; \$,  $p \leq 0.05$ .

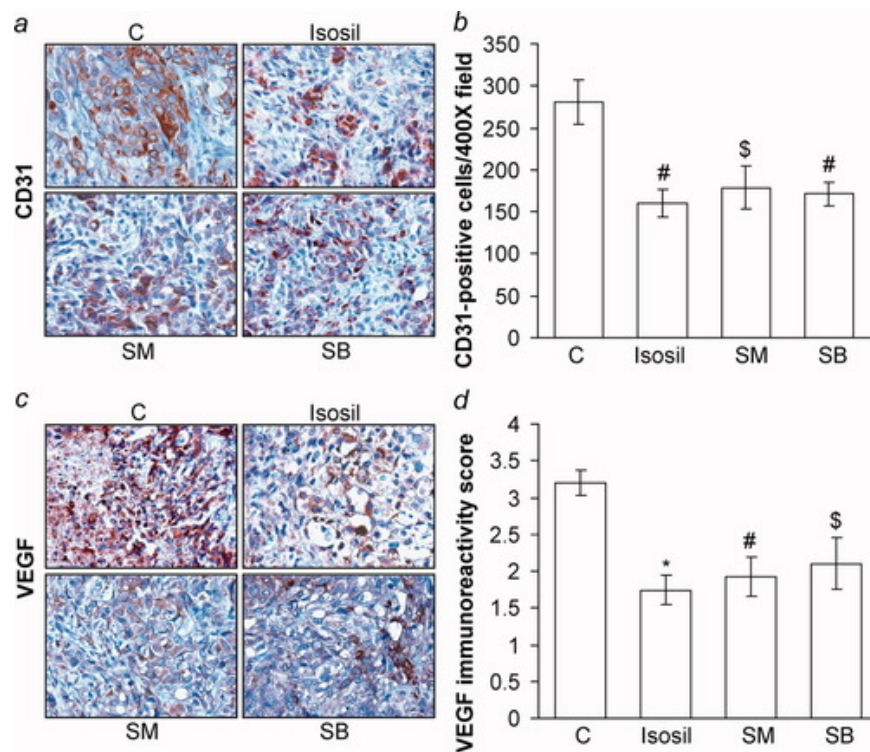
### Isosilibinin, silymarin and silibinin inhibit angiogenesis in DU145 xenograft

Earlier studies have shown that silymarin and silibinin possess strong anti-angiogenic activity,<sup>23-25</sup> so we next analyzed the tumors for CD31 staining to assess tumor microvessel density. The immunohistochemical analysis of tumor tissues (53 days treatment) showed that isosilibinin treatment decreased the CD31-positive (brown) cells by 43% ( $p \leq 0.01$ ), whereas silymarin and silibinin decreased by 36.2% ( $p \leq 0.05$ ) and 39.4% ( $p \leq 0.01$ ), respectively (Figs. 4a and 4b). There were no significant differences in the CD31-positive cells in tumors between different groups 24 days after treatment was discontinued (data not shown).

### Isosilibinin, silymarin and silibinin inhibit VEGF expression in DU145 xenograft

VEGF is an important angiogenic factor, which is secreted by tumor cells and regulate the tumor vascularization.<sup>26, 27</sup> Because we observed a strong decrease in tumor microvessel density with all 3 formulations, we next examined the tumor tissues for VEGF immunostaining. Results showed that 53 days of isosilibinin treatment resulted in 45.6% ( $p \leq 0.001$ ) decrease in VEGF immunoreactivity, whereas silymarin and silibinin treatment decreased the VEGF

immunoreactivity by 40% ( $p \leq 0.01$ ) and 34.4% ( $p \leq 0.05$ ) respectively (Figs. 4c and 4d). There was no statistically significant difference in VEGF staining between different groups 24days after the treatment was stopped (data not shown).

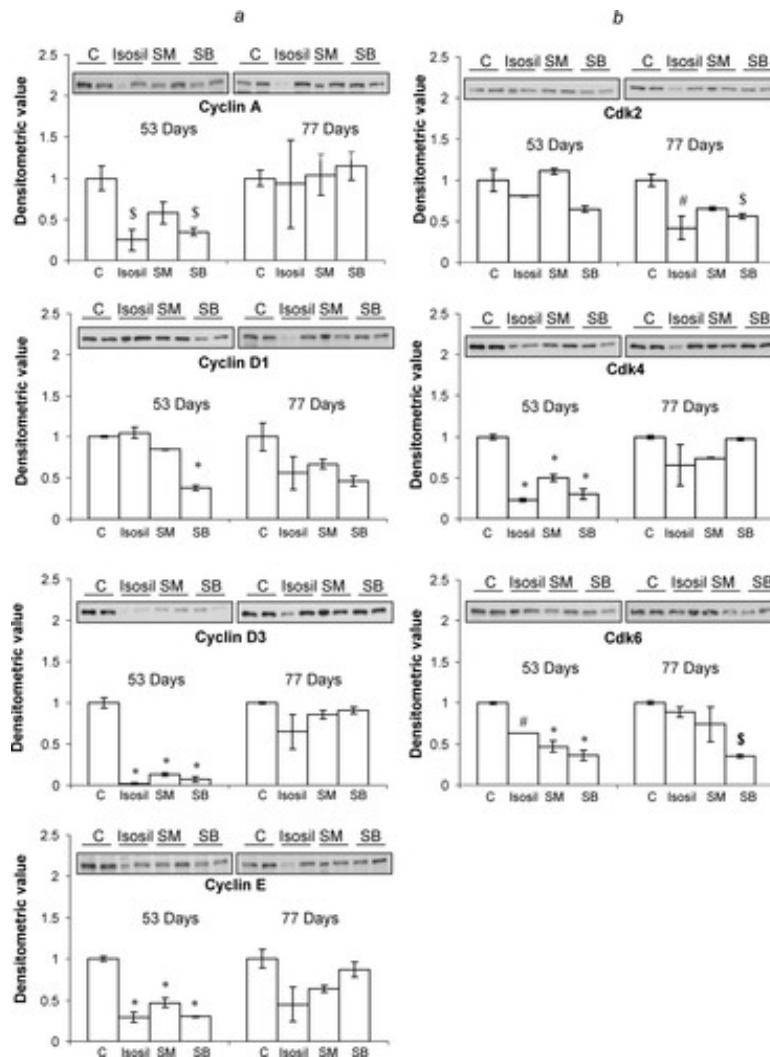


**Figure 4.** *In vivo* effect of isosilibinin, silymarin and silibinin on microvessel density and VEGF level in DU145 tumor xenografts in athymic nude mice. At the end of 53 days treatment, 7 mice from each group were sacrificed and tumor tissues were analyzed by immunohistochemical staining for CD31 (a, b) and VEGF (c, d) as detailed in the “Materials and methods”. For microvessel density CD31 positive cells were counted at  $\times 400$  field in 5 randomly selected areas in each tumor sample. VEGF immunoreactivity (represented by brown staining) was analyzed in 5 random areas for each tumor tissue and was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), 4+ (very strong staining). The data shown in the bar diagram represents mean and  $\pm$  SEM (error bars) of 4–5 samples for each group. Abbreviations: C, control; Isosil, isosilibinin; SM, silymarin; SB, silibinin. \*,  $p \leq 0.001$ ; #,  $p \leq 0.01$ ; \$,  $p \leq 0.05$ .

#### Isosilibinin, silymarin and silibinin differentially modulate cell cycle regulators in DU145 xenograft

The cell cycle is regulated by the interaction of various factors involving cyclins and cyclin-dependent kinases (Cdks).<sup>28-30</sup> Cell cycle deregulation has been considered as hallmark of cancer cells and is 1 of the factors underlying their unlimited replicative potential.<sup>31</sup> Next, we examined the effect of these compounds on the expression of various cell cycle regulators in both set of tumor tissues (53 days and 77 days) by Western blot analysis. Results showed that these compounds have differential effects on the expression of cell cycle regulators (Figs. 5a and 5b). Isosilibinin and silibinin significantly decreased cyclin A levels ( $p \leq 0.05$ ), whereas silymarin had no significant effect on cyclin A levels (Fig. 5a). Silibinin treatment inhibited cyclin D1 expression ( $p \leq 0.001$ ), whereas isosilibinin and silymarin treatment did not have any significant effect on cyclin D1 levels (Fig. 5a). All three compounds significantly inhibited the cyclin D3 and cyclin E levels in tissues from 53 days experiment ( $p \leq 0.001$ ) (Fig. 5a). None of the

formulations significantly affected the levels of cyclins (A, D1, D3 and E) in tumor tissue from the 77 days experiment (24 days treatment-free; Fig. 5a).



**Figure 5.** Effect of isosilibinin, silymarin and silibinin on cell cycle regulatory molecules in DU145 tumor xenografts in athymic nude mice. Two randomly selected samples from each group in 53 days and 77 days xenograft study were analyzed for cell cycle regulatory molecules. Lysates were prepared and Western blot analysis was performed for (a) cyclin A, cyclin D1, cyclin D3 and cyclin E, (b) Cdk2, Cdk 4 and Cdk6 as detailed in the “Material and methods.” The experiment was repeated at least once with fresh set of samples. The densitometric value of each band was analyzed by the Scion Image program and represented as a bar diagram. The data shown in the bar diagram represents mean and  $\pm$  SEM (error bars) of 4 samples for each group. Abbreviations: C, control; Isosil, isosilibinin; SM, silymarin; SB, silibinin. \*,  $p \leq 0.001$ ; #,  $p \leq 0.01$ ; \$,  $p \leq 0.05$ .

These formulations also targeted the expression of Cdks in the tumor tissue. Treatment with all the 3 of the formulations suppressed the expression of Cdk4 and Cdk6, but did not affect the Cdk2 level in the 53 days tumor tissues ( $p \leq 0.001$  to  $p \leq 0.01$ ) (Fig. 5b). The isosilibinin and silibinin groups had significantly lower expression of Cdk2 only after 24 days of withdrawal of treatment ( $p \leq 0.001$  to  $p \leq 0.05$ ) (Fig. 5b). Only silibinin treatment was effective in inhibiting the Cdk6 level in the 77 days tumor tissue ( $p \leq 0.05$ ) (Fig. 5b).

## Discussion

Phytochemicals based cancer management has been suggested as an attractive strategy against PCa, 1 of the leading cause of cancer-related mortality and morbidity around the world especially in the western countries.<sup>1, 7, 32, 33</sup> Silymarin, also known as milk thistle extract, has shown promise and potential as an ideal anti-cancer agent.<sup>12, 34</sup> Silymarin has been traditionally known for its hepatoprotective properties and used for reducing the toxicity related with alcohol, metals, mycotoxins, chemotherapy, radiotherapy, *etc.*<sup>35</sup> Extensive research within the last decade has shown that silymarin and its active semi-purified fraction silibinin inhibit PCa growth and progression in both *in vitro* and *in vivo* models.<sup>12, 13, 25, 30</sup> Silymarin and silibinin have been shown to inhibit the PSA secretion by PCa LNCaP cells by targeting the function of androgen receptor.<sup>36, 37</sup> The anti-tumorigenic action of silymarin has been related with its inhibitory effect on erbB1 receptor signaling.<sup>38</sup> Studies have also shown that silymarin and silibinin activate cellular checkpoints and Cdk inhibitors along with inhibition of Cdks activity, thus halting the cell cycle to either G1 and/or G2M phase.<sup>30, 38, 39</sup> Silymarin treatment has been reported to inhibit the 3,2-dimethyl-4-aminobiphenyl (DMAB)-induced prostate carcinogenesis in male F344 rats.<sup>40</sup> Silibinin has been shown to inhibit the growth of PCa cells in xenograft studies as well as in the transgenic adenocarcinoma of the mouse prostate model.<sup>13, 25, 41</sup> As a result of these preclinical studies, the efficacy of silibinin (as the Siliphos formulation with phosphatidylcholine) is being evaluated in Phase II clinical trial in PCa patients, after the successful completion of Phase I trial.<sup>42</sup> Despite this progress in milk thistle research, the relative contribution of other constituents of silymarin to its efficacy remains largely unknown.

The present is the first study to demonstrate the *in vivo* growth inhibitory potential of isosilibinin, a diastereoisomeric two-compound formulation from silymarin. The present study clearly showed that isosilibinin treatment inhibited advanced human PCa DU145 xenograft growth in athymic nude mice, which was equally good, when compared with silymarin or relatively better (without statistical significance) when compared with silibinin. One particularly notable finding of the present study is that even after 24 days of withdrawal of the treatment agents, tumor volumes remain decreased in isosilibinin, silymarin and silibinin treated groups, suggesting that there would not be a compensatory tumor growth even after withdrawal of these compounds. Earlier studies have shown the excellent safety profile of silymarin and silibinin, and both are known to be nontoxic even at higher doses.<sup>42-44</sup> In fact, rodent LD<sub>50</sub> values have never been achieved for silibinin or silymarin. The present study illustrates for the first time the nontoxic nature of isosilibinin at the dose level used.

The study of cancer-related biomarkers has been suggested as a critical element in understanding the clinical relevance of chemopreventive agents.<sup>45, 46</sup> In the present study, we focused on 3 surrogate biomarkers, namely cell proliferation, apoptosis and angiogenesis, which are widely used and linked to the growth and progression of cancer, including PCa.<sup>25, 45, 47</sup> The level of cellular PCNA is regarded as 1 of the markers for cellular proliferation.<sup>48</sup> PCNA is a 36 kDa auxiliary protein to DNA polymerase and it was identified as an antigen that is expressed in the nuclei of cells during the DNA synthesis phase of cell cycle. In the present study, isosilibinin, silymarin and silibinin treatment each significantly inhibited the PCNA levels in xenograft tissue. These results are correlated with the decreased tumor volume observed with the treatment of these formulations.

Apoptosis evasion has been suggested as 1 of the hallmark of cancer cells.<sup>31</sup> Advanced human PCa including DU145 cells are known to have constitutively active survival signaling pathways, rendering these cells apoptosis-resistant.<sup>49</sup> Treatment with isosilibinin, silymarin or silibinin significantly increased the apoptotic cell population in the xenograft tissue as measured by TUNEL staining. Even though this might not be the main mechanism of action for these compounds, as apoptotic cell population remained less than 5% of total cells, it could be contributing partly to their overall antitumor effects.

Angiogenesis is 1 of the important elements in the process of tumorigenesis and is considered essential for the growth and progression of cancer cells.<sup>45, 50, 51</sup> It has been reported that angiogenesis is especially critical for the growth and progression of solid tumors, as tumor growth beyond 2–3 mm size is often preceded by increased formation of new blood vessels.<sup>45, 51</sup> Therefore, targeting angiogenesis represents 1 of the most promising approaches to control tumor growth.<sup>45</sup> The present study revealed that isosilibinin followed by silibinin and silymarin were effective in inhibiting the microvessel density in the tumor xenograft, suggesting their antiangiogenic efficacy against advanced PCa. Studies have shown that process of neo-angiogenesis is highly complex and tightly regulated by multiple pro-angiogenic and anti-angiogenic factors. One of the important pro-angiogenic factors is VEGF, which is expressed and secreted by advanced cancer cells and promotes endothelial cell proliferation, migration and differentiation to initiate as well as maintain tumor angiogenesis.<sup>26, 27</sup> In the present study, isosilibinin, silymarin and silibinin treatment strongly inhibited VEGF expression in the tumor tissue, which might contribute to the decreased microvessel density observed with their treatments.

Aberrant cell cycle regulation has been recognized as the characteristic of cancers including PCa.<sup>31</sup> Earlier studies have suggested cell cycle control as the basis for cancer chemoprevention through phytochemicals and dietary agents.<sup>52, 53</sup> Therefore, cancer chemopreventive agents have been routinely screened for their efficacy to target cell cycle regulatory determinants in cancer cells. We have reported that silymarin and its constituents regulate the expression of various cell cycle regulatory molecules and cause cell cycle arrest in PCa cells.<sup>18, 30</sup> The present *in vivo* data also confirms that these formulations have differential effects on the expression of cell cycle regulatory molecules. These studies further confirm that these formulations, even though closely related chemically, might have subtle differences in their mechanism of action; but further studies are warranted to clearly understand these aspects.

In conclusion, the present study is the first to demonstrate that isosilibinin treatment inhibits the growth of advanced human PCa cells *in vivo* without any toxicity. This potency of isosilibinin was either equal to or better than silymarin/silibinin. Isosilibinin was also shown to possess *in vivo* anti-proliferative, anti-angiogenic, pro-apoptotic and cell cycle modulatory properties. Further studies are needed to clearly understand the mechanism of action for isosilibinin alone or isosilibinin-enriched silymarin formulations for their clinical utility against PCa.

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